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Application of Proteomics to Elucidate the Mechanism of Toxicity of the Chemical Warfare Agent Sulfur Mustard

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14. ABSTRACT
The complex molecular and cellular changes that occur following exposure to sulfur mustard (SM) have made it difficult to elucidate the primary mechanisms associated with SM-induced skin vesication. This report describes the use of proteomic approaches and methodologies to address this problem. Proteomic technologies, primarily two-dimensional (2D) gel electrophoresis and mass spectrometry, allow monitoring and identification of the complex molecular changes that occur in cells following toxic exposures. Cultured human epidermal keratinocytes were exposed to a vesicating dose (200 mM) of SM for up to 24 hours, the cellular proteins were extracted, and were analyzed by 2D electrophoresis. Selected differentially expressed proteins were analyzed by mass spectrometry for identification. Using this approach we have identified proteins involved in cytoskeletal structure as potential targets of SM. We are applying these techniques to identify protein pathways involved in the molecular mechanism of SM-induced vesication, and to identify potential therapeutic targets for the development of vesicant medical countermeasures.

15. SUBJECT TERMS
Sulfur mustard, proteomics, vesicant, two-dimensional gel electrophoresis, mass spectrometry, keratinocyte

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INTRODUCTION

Multiple cellular pathways have been investigated to elucidate the mechanism of toxicity of the chemical warfare agent sulfur mustard (SM). These pathways have included the loss of important enzyme cofactors such as glutathione (Gross et al., 1993), the activation of proteases (Cowan et al., 1991; 1993), the loss of energy storage molecules such as ATP (Meier et al., 1996) and NAD (Papirmeister et al., 1985), the activation of apoptosis (Smith et al., 1997; Rosenthal et al., 1998), the loss of Ca^{2+} regulation (Ray et al., 1995), the loss of cell cycle regulation (Smith et al., 1993), and cytokine production (Schlager et al., 2002; Ricketts et al., 2000; Sabourin et al., 2000; Arroyo et al., 1999; Lardot et al., 1999; Tsuruta et al., 1996; Rikimaru et al., 1991). Evidence suggests that many of these cellular pathways contribute to SM-induced cellular and tissue damage. Because SM is a potent alkylating agent, biological molecules such as DNA, RNA and protein can be modified through electrophilic reaction with the ethylene episulfonium intermediate. Thus, direct effects on production or functional quality of specific structural proteins or enzymes may also be important (Smith et al., 1995).

In the past, molecular and cellular biology techniques allowed investigators to focus on only one or a small number of proteins at a time. However, large-scale genome sequencing projects, such as the Human Genome Project, are revolutionizing applied biology and toxicology. There is a move away from a reductionist approach of studying one gene or protein at a time toward a more global approach of studying molecular and cellular networks and how these networks integrate information and respond to the environment (Farr and Dunn, 1999). Recent technological developments allow researchers to study the function of a single gene or protein in the context of cellular and molecular networks, or to study the response of numerous genes or proteins to an environmental stimulus (e.g., SM exposure). These new molecular techniques allow for higher throughput studies and are well suited to elucidating the mechanisms of cellular responses to environmental stimuli (e.g., SM exposure) (Steiner and Anderson, 2000).

Proteomics is an emerging discipline focused on large-scale identification of changes in protein expression, post-translational modifications, and protein function (for review see Blackstock and Weir, 1999). Proteomics is the study of the proteome, which is the total set of proteins expressed in a given cell or tissue at a given time. Most of the primary technology used in the field of proteomics was developed decades ago, but has been refined considerably in recent years. The first part of the proteomics technology, two-dimensional gel electrophoresis (2D electrophoresis), has remained the protein separation method with the highest resolution and sensitivity to date (O'Farrell, 1975; Klose, 1975). Two-dimensional gel electrophoresis combines isoelectric focusing in the first dimension, which separates proteins on the basis of their isoelectric points (charge), with conventional SDS polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, which separates proteins on the basis of their molecular weight (size). For several years 2D electrophoresis suffered from problems with reproducibility. However, recent advances in isoelectric focusing technology have greatly improved the reproducibility and reliability of 2D electrophoresis (Gorg et al., 1985; Gorg et al., 1988).

The second part of the proteomic technology is mass spectrometry (MS). Recently, MS techniques and sample preparation protocols have been developed that allow analysis of very small quantities of proteins, down to the femtomole level (Yates, 2000). Protein spots from a 2D

gel can be excised and digested with a protease, such as trypsin, to generate peptide fragments of the protein of interest. These peptides are then extracted and analyzed by MS using one of two main technologies, which are based on the method of sample ionization (Patterson and Abersold, 1995). Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) MS uses a laser pulse to desorb and ionize a protein sample that has been co-crystallized with an energy-absorbing matrix chemical. The ions are accelerated down a flight tube, and the time-of-flight of an ion to the detector is proportional to its mass. Thus, the mass-to-charge ratios of each of the peptide fragments from a tryptic digest can be accurately determined. This information is used to query a database that runs a simulated trypsin digest on known proteins in the protein sequence databases and attempts to match the "peptide mass fingerprint" of the unknown protein with the peptide mass fingerprint of a known protein. Using the mass accuracy now available, this technique alone is often sufficient to characterize proteins. If a match is not found, then electrospray ionization tandem MS can be employed. In this technique, ions are formed using an electrospray source, and a triple quadrupole MS instrument can then be used to fragment the peptide ions and analyze the resulting product ions. This form of MS is used to generate additional partial sequence information (Mann and Wilm, 1994). This information can complement the mass data and be used to search protein and expressed sequence tag databases with extremely high specificity.

We are currently using these techniques to identify protein changes in human epidermal keratinocytes (HEK) in response to a vesicating dose of SM. Our data reveal changes over time in the expression of HEK proteins, as well as changes in the proteins secreted by HEK in response to SM exposure. The overall objective of this work is to develop a database of pathophysiological protein changes induced by SM. This information will help us understand the mechanism of SM-induced damage at the cellular and molecular level, and aid efforts to formulate pretreatment and treatment strategies.

MATERIALS AND METHODS

Experimental Design.

An overall outline of the methodology used in proteomics and its application to investigating SM is shown in Figure 1.

Cell culture and SM exposure.

Adult human epidermal keratinocytes (HEK) were obtained as cyropreserved first passage (P1) stocks of 5 x 10^5 cells per vial from Clonetics (San Diego, CA). Cells were thawed and seeded at 2,500/cm^2 into 75 cm^2 flasks (Corning). HEK were grown in serum-free keratinocyte growth medium (Clonetics) to 70-80% density prior to trypsin detachment and reseeding at 2,500/cm^2 into 150 cm^2 flasks. Third passage flasks of HEK at 70% confluence were removed from the 37°C culture incubator and allowed to cool to room temperature prior to treatment. A frozen aliquot of SM in modified Eagle's medium (SM-MEM) was thawed, and when the two-liquid phases were apparent by visual inspection of the SM droplet, a 4 mM SM solution was made by rapid liquid vortex. The solution was placed in an ice bath to slow the rate of hydrolysis. For each time point investigated, one to three flasks of HEK were either left

untreated (control) or exposed to 200 μM SM. This dose of SM is considered sufficient to produce a blistering event if the same dose is exposed to skin (W.J. Smith, personal communication). HEK were exposed to SM for 0 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, and 16 hr.

Isolation of cellular and secreted proteins.

After exposure to SM, the cell culture media was collected. The HEK were washed twice with Hanks balanced salt solution. The cells were solubilized in 3 ml of 2D lysis buffer (9M urea, 4% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate), 2M thiourea, 0.5% Pharmalytes (Amersham Pharmacia Biotech, Piscataway, NJ), 10 mM dithiothreitol (DTT) and 40 mM Tris). These samples contain cellular HEK proteins and were stored at –80°C until use.

To examine proteins secreted by HEK into the culture media, the collected cell culture media was centrifuged at 1,000 x g for 10 minutes to remove any cells. The proteins were precipitated by incubation with 2 volumes of ice-cold acetone at –20°C for 2 hours. The precipitated proteins were collected by centrifugation at 10,000 x g for 40 minutes at 4°C. The protein pellet was resuspended in water, and the proteins were precipitated again as described. The protein pellet was resuspended in 2D lysis buffer. These samples contain secreted HEK proteins and were stored at –80°C until use.

Two-dimensional gel electrophoresis.

Samples were thawed to room temperature and spun at 14,000 rpm in a microfuge for 10 minutes before use. Protein concentrations were determined using the Coomassie Plus protein assay reagent (Pierce Chemicals, Rockford, IL) according to the manufacturer's instructions. Protein lysate was diluted into rehydration buffer (9.0 M urea, 2% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate), 0.5% IPG buffer (Amersham Pharmacia Biotech, Piscataway, NJ), 18 mM DTT) and fractionated by isoelectric focusing using Immobiline Dry-Strip gels on an IPGphor isoelectric focusing apparatus (Amersham Pharmacia Biotech, Piscataway, NJ) for 70,000 volt-hours. After focusing, the strip gels were prepared for loading onto conventional SDS-PAGE gels by incubation in SDS equilibration buffer (50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 65 mM dithiothreitol for 15 minutes at room temperature. The strip gels were then incubated in equilibration buffer containing 135 mM iodoacetamide. The equilibrated strip gels were then loaded onto large format (23x20 cm) gels and run at 100 V for 14.5 hours (DALT system, Amersham Pharmacia Biotech). The proteins were then visualized by protein staining. For silver staining, the protocol for fast silver staining as described by Rabilloud (1999) was employed. For colloidal coomassie blue staining, a stock staining solution of 10% ammonium sulfate, 2% phosphoric acid (85%), and 0.1% colloidal coomassie blue G-250 (Bio-Rad, Hercules, CA) made as a 5% stock in water, was diluted 4:1 with methanol to produce gel staining solution. After electrophoresis, gels were rinsed briefly in water and then stained for three to four hours in gel staining solution and destained in water. Images of each 2D gel were captured using a Kodak DC120 zoom digital camera and Kodak Digital Science™ electrophoresis documentation software (Eastman Kodak,

Rochester, NY) or an Umax PhotoLook III digital scanner (Umax, Fremont, CA) driven by Adobe Photoshop software (Adobe Systems, San Jose, CA).

In-gel digestion of proteins.

Selected proteins were excised from the stained gel and prepared for in-gel digestion (Rosenfeld et al., 1992). The gel pieces were destained with three changes of 25 µl 50 mM ammonium bicarbonate/25 µl acetonitrile. The gel pieces were reswelled in 25 µl 50 mM ammonium bicarbonate, dehydrated in acetonitrile and dried by vacuum centrifugation in a Speed Vac (Savant Instruments, Holbrook NY). The protein in each of the dried gel pieces was reduced by rehydration in 10 mM DTT in 100 mM ammonium bicarbonate and incubation at 56°C for one hour. The protein was then alkylated by treatment with 55 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 45 minutes. The treated gel pieces were washed in acetonitrile, 50 mM ammonium bicarbonate, acetonitrile and then dried by vacuum centrifugation. The gel pieces were rehydrated on ice for 45-60 minutes in 25 µl of 10 ng/µl trypsin (Worthington, Lakewood, NJ) in 50 mM ammonium bicarbonate. Excess trypsin was discarded; the gel pieces were covered with 50 mM ammonium bicarbonate and incubated overnight at 37 °C. Each digest was recovered and pooled with several aqueous (water) and organic (50% acetonitrile/5% formic acid) extractions of the same gel piece. The volume of each pooled digest was reduced to 10 µl by vacuum centrifugation. Each digest was dried and resuspended in 10 µl of water. Two to five µl of 50% acetonitrile/5% formic acid was added, and the digest was further purified using reversed-phase ZipTips (pipette tips containing C18 chromatography media; Millipore, Bedford, MA) according to the manufacturer's instructions.

Mass spectrometry analysis.

One µl of the digest was spotted on a sample plate with 1 µl of matrix preparation (10 mg/ml α-cyano-4-hydroxycinammic acid (Sigma Chemicals, St. Louis, MO) in 50% acetonitrile/1% trifluoroacetic acid) and allowed to air-dry. The sample was analyzed using a Voyager STR Biospectrometry Workstation MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). A tryptic mass fingerprint was generated from the masses of the peptide fragments. This information was used to query the OWL and National Center for Biotechnology information non-redundant (NCBInr) protein databases using the Mascot search engine (Perkins et al., 1999).

RESULTS

HEK were exposed to 200 µM SM for 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr and 16 hr. The cells were collected and lysed. The cellular proteins were separated via 2D electrophoresis. The proteins were visualized by silver staining (Figure 2). A variety of proteins changes were observed over time in response to SM exposure, including upregulated and downregulated expression of specific proteins. One cellular protein in particular of approximate molecular weight 29 kD and pI of 5.5 (indicated by a red arrow) is not expressed in control HEK, but is induced between 1 and 2 hr after SM exposure and is highly expressed through 16 hr after SM exposure. The expression level of many proteins does not appear to change in response to SM exposure, suggesting that a global up- or down-regulation of protein expression is not occurring.

We were also interested in identifying proteins that are secreted by HEK in response to SM exposure. It has been shown that proteases and inflammatory mediators, many of which are secreted proteins, may play an important role in SM-induced vesication (Cowan et al., 1993; Arroyo et al., 1999). To examine HEK proteins secreted in response to SM exposure, we collected the media from exposed HEK, precipitated the proteins, and fractionated the secreted proteins via 2D electrophoresis. The proteins were visualized by silver staining (Figure 3). Similar to what was seen with cellular HEK proteins in response to SM exposure, a variety of protein changes were observed 16 hr after SM exposure. The protein changes were primarily upregulation of the levels of a number of proteins.

To further characterize proteins that are altered or upregulated in response to SM exposure, we excised proteins from two gels (spots indicated in Figure 4) and digested them with trypsin. These proteins are altered rapidly (15 minutes) after exposure to 200 μM SM (Figure 4). The tryptic fragments were analyzed by MALDI-TOF mass spectrometry and peptide fingerprints were generated (Figure 5). Analysis of the mass spectra in Figure 5A revealed a variety of peaks corresponding to peptide fragments. Searching of the NCBI protein database revealed that the peptides match to human beta-tubulin with sequence coverage of 43% (Figure 5A). Similarly, analysis of the mass spectra in Figure 5B revealed a variety of peaks that when searched using Mascot, identified human alpha-tubulin, with sequence coverage of 22% (Figure 5B).

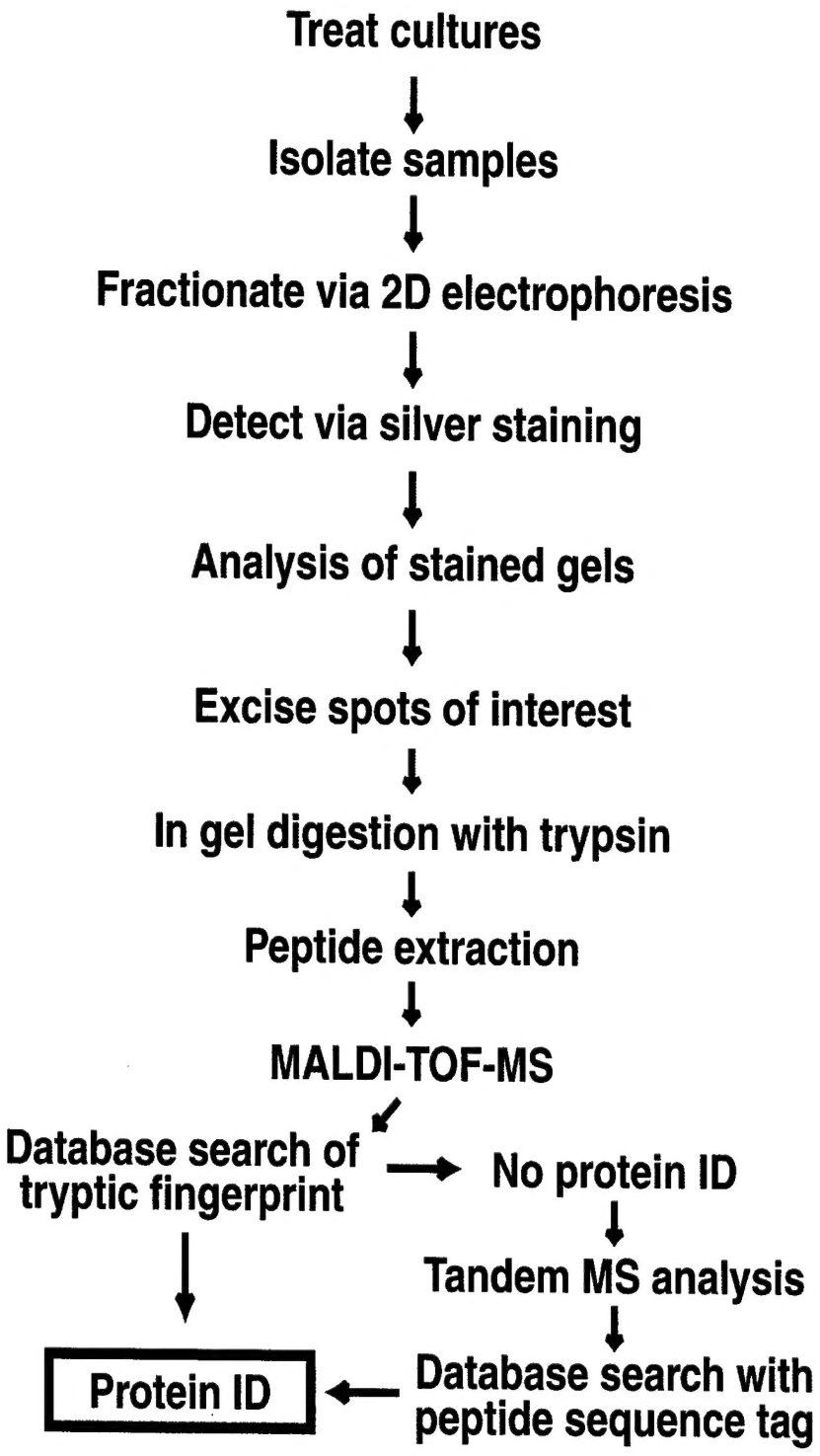

Figure 1: A flowchart depicting the use of proteomics to investigate sulfur mustard toxicity.

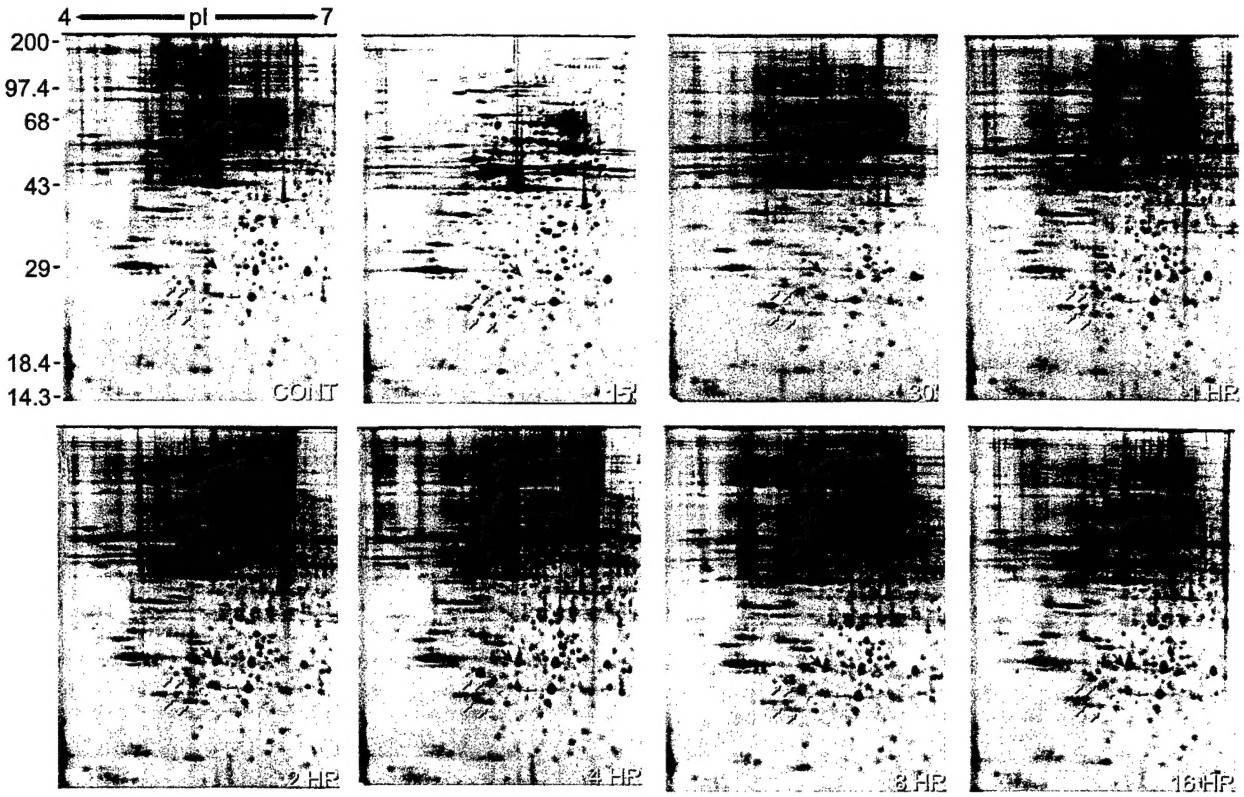

Figure 2: 2D gels of HEK exposed to sulfur mustard. HEK were exposed to SM for the times indicated and lysed in 2D lysis buffer. Cell lysates were fractionated by 2D gel electrophoresis (these samples were fractionated in parallel under identical conditions). The proteins were separated in the first dimension on the basis of isoelectric point (charge, pI) using a linear pH gradient of pH 4-7 (indicated above the CONTROL gel). Proteins were then separated in the second dimension on the basis of molecular weight (size) using a 10% acrylamide SDS-PAGE gel. Molecular weight markers are indicated to the left of the CONTROL gel. Proteins were visualized by silver staining. Arrows indicate proteins that change over time in response to SM. A protein (~29 kD, pI 5.5) that is highly induced in response to SM is indicated by a large arrowhead. A representative sample of altered proteins is indicated by the smaller arrows. The overloaded region of the gel between molecular weight 43 kD and 66 kD contains the keratin proteins, actin proteins, and tubulin proteins that are in high abundance in HEK.

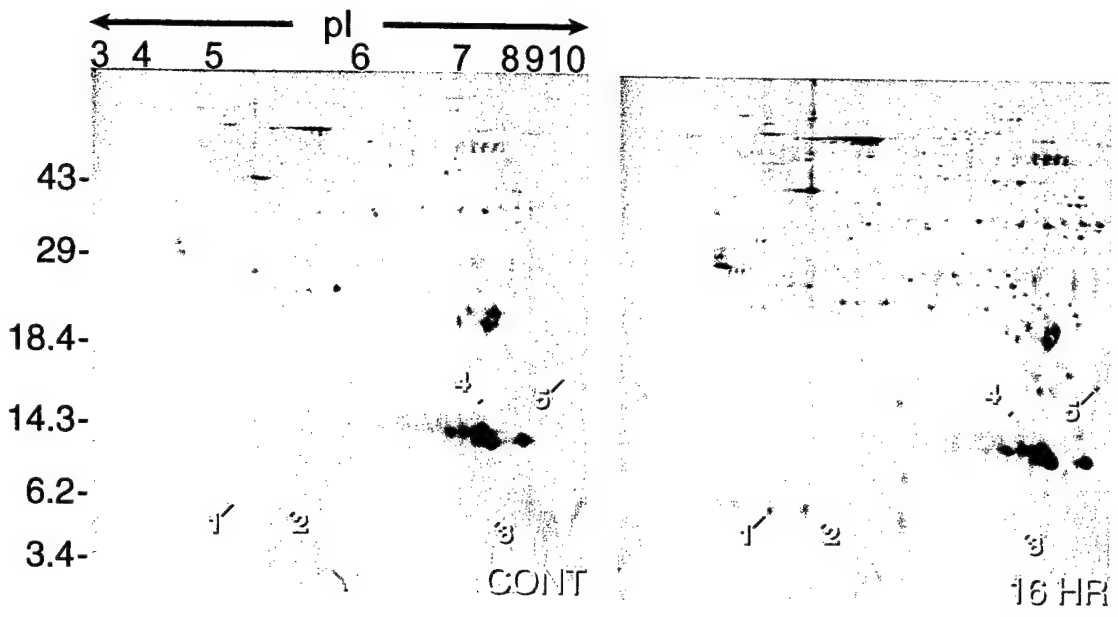

Figure 3: 2D gels of proteins found in the media of HEK exposed to sulfur mustard. Media from SM-exposed HEK was collected, and the proteins were precipitated and solubilized in 2D lysis buffer. The protein was fractionated by 2D electrophoresis. A nonlinear pH gradient of pH 3-10 was used, indicated above the CONTROL gel. Molecular weight markers are indicated to the left of the CONTROL gel. Separated proteins were visualized by silver staining. Numbers indicate a representative subset of proteins whose secretion is altered by exposure to SM.

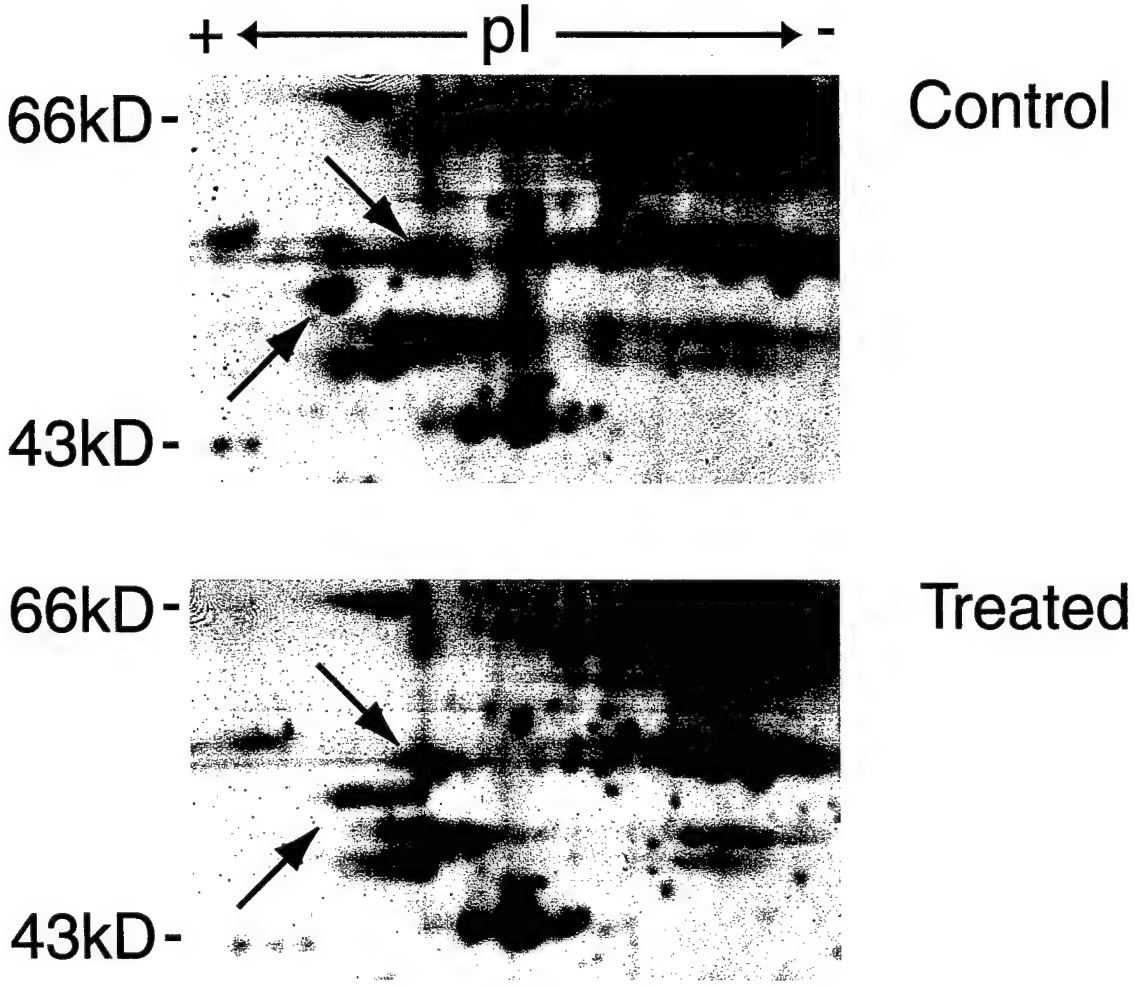

Figure 4: Alterations in HEK proteins after exposure to vesicating concentrations of sulfur mustard. HEK were exposed to 200 uM HD for 15 min and lysed in 2D lysis buffer. Cell lysates were fractionated by 2D gel electrophoresis using a pH gradient of pH 4-7 and a 10% acrylamide SDS-PAGE gel. The proteins were visualized by colloidal coomassie blue staining. A portion of the gel is shown to highlight changes in specific proteins, indicated by the arrows. These proteins were subjected to in-gel digestion and mass spectrometry analysis.

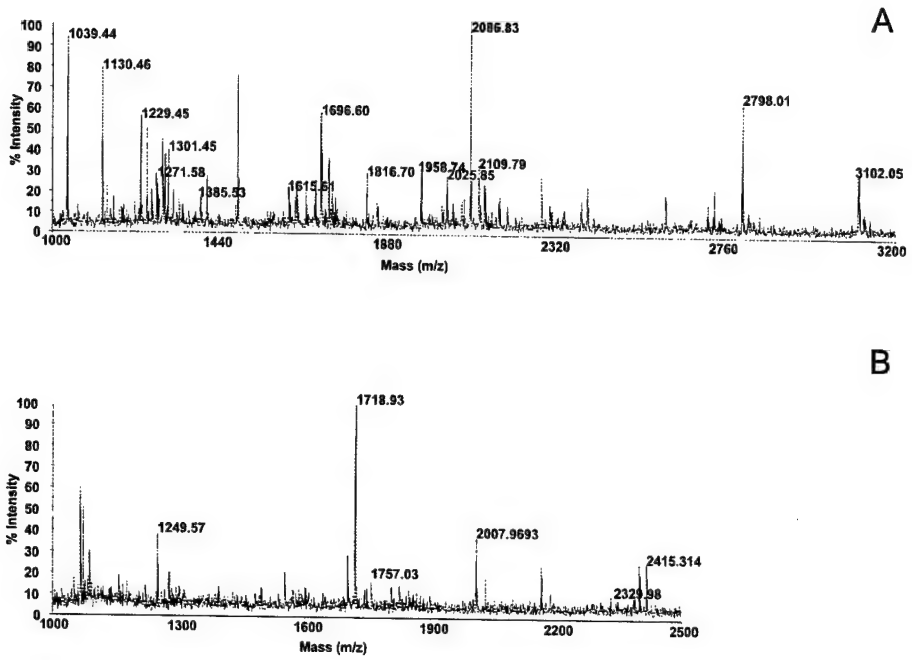

Figure 5: Identification of α-tubulin and β-tubulin as proteins altered after exposure of HEK to SM. The proteins indicated in Figure 4 were excised from the gel, digested to tryptic peptides with trypsin, extracted from the gel, and analyzed by MALDI-TOF mass spectrometry. Panel A shows the mass spectrum of the peptides obtained from the protein identified by the down arrow in Figure 4. The labeled peaks indicate peptide masses that match to human beta-tubulin, and provide 43% amino acid sequence coverage of the protein. Panel B shows the mass spectrum of the peptides obtained from the protein identified by the up arrow in Figure 4. The labeled peaks indicate peptide masses that match to human alpha-tubulin, and provide 22% amino acid sequence coverage of the protein.

DISCUSSION

HEK undergo complex molecular changes in response to SM exposure. We are applying the proteomic techniques of 2D electrophoresis and mass spectrometry to begin identifying changes in the protein profiles of HEK exposed to SM. Our results reveal a variety of changes in response to SM exposure. We have found changes in the HEK protein profiles of both HEK cellular proteins as well as secreted proteins using 2D electrophoresis. Using mass spectrometry techniques we have begun to positively identify these proteins with peptide mass fingerprinting and database interrogation. Work is currently underway to further characterize the proteins that are altered in response to SM exposure.

These proteomic techniques provide a way to identify global changes in protein expression, modification and function. Proteomic approaches have some advantages over global mRNA-based approaches to identify changes in gene and protein expression. One advantage is that the levels of mRNA and protein do not always correspond in a 1:1 ratio (Anderson and Seilhamer, 1997). Often, mRNA levels and protein levels can vary for the same gene product. Another advantage of a proteomic approach is that one can look at protein modifications, protein-protein interactions, and protein localization in various cellular compartments, all of which have a significant impact protein function. mRNA-based approaches cannot assess these types of changes in protein function.

However, proteomic approaches do face some challenges. One important challenge is the wide dynamic range of protein expression in a cell. This makes it difficult to detect low abundance proteins. We have encountered this problem with our HEK model, and are developing methods to enrich different subcellular fractions to circumvent this problem. Another challenge is scaling up for high-throughput studies of many proteins identified by 2D electrophoresis. To address this problem, we are working to develop focused functional proteomic approaches, in which our proteomic analysis is driven by known functional changes (e.g., changes in phosphorylation, changes in protein secretion). This will allow us to focus our proteomic efforts in a function-based way.

Using these techniques, we will assemble a database of pathophysiological protein changes in response to SM. This information will help us understand the molecular mechanism of SM toxicity, and aid in the development of pretreatment and treatment strategies to counteract the use to this chemical warfare agent.

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